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Stratum corneum hydration : mode of action of moisturizers on a molecular level

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Chapter 6

In vivo and in vitro interaction of lipophilic moisturisers with stratum corneum lipids

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1. Summary

Dry skin symptoms such as scaling and itching are often treated with lipophilic moisturisers. The aim of this study was to investigate the effect of lipophilic moisturisers on the stratum corneum ultra-structure.

Lipophilic moisturisers were applied on the forearms of four healthy volunteers for three hours. Subsequently, the application sites were tape-stripped, and selected tape-strips prepared for Freeze Fracture Electron Microscopy (FFEM), a method to visualise the SC intercellular lipid parallel to the skin surface. Isolated stratum corneum was pre-treated with the same lipophilic moisturisers for 24 hours prior to performing Small Angle X-Ray Diffraction (SAXD) measurements, to investigate the effect on the lipid lamellae. Additionally, the lipid organisation of mixtures prepared with ceramides, cholesterol, free fatty acids and lipophilic moisturiser in a 2:1:1:1 molar ratio were studied using SAXD.

The FFEM data (in vivo) as well as the SAXD data (in vitro) show that the lipophilic moisturisers do not change the lipid lamellar organisation in the stratum corneum. Addition of 20% m/m lipophilic moisturiser to the ceramide:cholesterol:free fatty acids mixture did not

inhibit the formation of the long periodicity phase (LPP), the characteristic lamellar phase in stratum corneum, even though there was clear evidence that two of the three moisturisers were at least partially incorporated in the LPP. Concluding, all findings suggest that the lipophilic moisturisers investigated in this study do not drastically change the lamellar organisation of the SC intracellular lipid matrix, but that the moisturisers form separate domains in stratum corneum, as was visualised by FFEM.

2. Introduction

Symptoms of dry skin such as scaling, itching and irritation are very common occurrences, which find their cause in the stratum corneum (SC). This uppermost layer of the skin is essentially dead. However, even though it is not a viable tissue, many enzymatic processes still occur in this part of the skin (Harding, 2000). As the enzymatic processes are affected by the hydration of the SC, an optimal water level in the SC is essential for proper functioning of the SC. This water level is retained in the SC by the so-called natural moisturising factor (NMF), which is produced by degradation of a SC protein (filaggrin) at a specific water level and consists of highly hygroscopic low molecular weight compounds in the corneocytes. SC water and NMF therefore work in synergy: An optimal water level in SC promotes NMF production, while the presence of NMF ensures water retention in the SC. Two other important SC processes also dependent on the water level are cornification and desquamation. The cornification process, in which the cell membrane of the corneocyte is converted from a normal membrane into a thick, hard protein wall covered with stratum corneum lipids is assembled by transglutaminases (Hirao, 2003), while desquamation is most likely regulated by the activity of several enzymes such as stratum corneum chymotryptic enzyme (SCCE) and cathepsin D (Harding, 2000). All these enzymes only have optimal activity at the optimal water level and pH in SC.

A suboptimal enzyme activity will lead to classical symptoms of dry skin: scaling, itching and irritation. These symptoms are generally treated with hydrophilic (e.g. glycerol, urea) and/or lipophilic (e.g. petrolatum) moisturisers (Rawlings et al., 1994a).

To repair dry skin it is essential to increase the hydration of the SC. This can be achieved by applying hydrophilic or lipophilic moisturisers, or a combination of both. Hydrophilic moisturisers are usually low molecular weight, hygroscopic substances, which supposedly penetrate the SC and retain water, much like the NMF does. Lipophilic moisturisers, e.g. fatty acids, waxes or triglycerides, most likely achieve their effect by occluding the skin, hereby

preventing water evaporating from it. However, it has been shown that some lipophilic substances that have moisturising capabilities are not occlusive (Wiechers J.W., 1999). They may, however, have certain interactions with the SC lipid matrix, which increase their barrier function inside the SC, rather than on its surface. It is known from literature that substances, which have interactions with the SC lipids may modify its lamellar structure, which can have serious deleterious effects on the skin's barrier function, leaving the skin more penetrable to water and other agents (Pilgram et al., 2001a), (Suhonen et al., 1999a). In skin penetration, these enhancers (e.g. azones, oleic acid) are often used to increase the amount of a transdermally delivered drug. However, for an intended moisturiser, a penetration enhancing effect would be adverse, as it would not only increase water evaporation and reduce the water level in the SC, but would also increase the loss of NMF from the stratum corneum during bathing. A moisturiser should therefore modify the SC lipid structure to be less permeable to water.

In this study, we examined the effects of three lipophilic moisturisers, which were selected taking into account their physical properties. All three are liquid at physiological temperatures and are isostearic acid (i.e. a branched carbon backbone) derivatives. The first one, isostearyl isostearate (ISIS) is an ester-dimer of isostearic acid, while the second, isopropyl isostearate (IPIS) is an ester of isostearic acid and isopropanol, resulting in a molecule with a much shorter chain length. The third derivative, glycerol monoisostearate (GMIS), is an ester of glycerol and isostearic acid, combining a long, branched carbon backbone with a hydrophilic group.

We have studied the ultra structure of the SC after *in vivo* application of these three lipophilic moisturisers using freeze fracture electron microscopy (FFEM). Additionally, we have studied the effect of the moisturisers on the lipid lamellar phases after completely immersing isolated SC in the moisturisers using small angle X-ray diffraction (SAXD). Finally, the possibility of incorporation of the moisturisers into the lipid lamellae was studied using isolated SC lipids and SAXD.

3. Materials and Methods

Materials

Isostearyl isostearate (ISIS), isopropyl isostearate (IPIS) and glycerol monoisostearate (GMIS) were kindly supplied by Uniqema (Gouda, The Netherlands). Palmitic acid, stearic acid,

arachidic acid, behenic acid, docosatrienic acid, lignoceric acid, cerotic acid, and cholesterol were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). All organic solvents used were of analytical grade and manufactured by Labscan Ltd (Dublin, Ireland). The Avery T-406 adhesive was a kind gift from Avery Dennison (Turnhout, Belgium). All other chemicals used were of analytical grade and all solutions were prepared in Millipore water.

In vivo application of moisturiser and tape-stripping

Using the tape-stripping technique, SC was obtained from the ventral forearms of healthy volunteers. At least four volunteers were used for each moisturiser. The volunteers were asked not to apply any pharmaceutical or cosmetic product on their ventral forearms within 24 hours of the start of the study. None of the volunteers had a history of skin disease, or hypersensitivity towards skin care products.

Thirty minutes before the start of the experiment, the skin of the ventral forearm was cleaned with a 70% alcohol wipe. 30 µl of a neat moisturiser was applied onto a 1 cm^2 site on the forearm and left non-occlusively for 3 hours. An untreated site served as a control.

Immediately after treatment, the stratum corneum was stripped. Tape (scribble paper covered with Avery T-406 adhesive (Avery, Turnhout, Belgium) was pressed on the skin surface and was removed with one fluent stroke. The 1st, 5th, 15th and 25th tape-strips were processed for freeze fracture electron microscopy as described below.

As a reference to the tape-stripping depth, TEWL measurements (Tewameter TM 210, Courage + Kazaka, Cologne, Germany) were performed before application and after each tape-strip processed for FFEM.

Freeze fracture electron microscopy

Tape-strips were cut into small pieces of approx. 1 mm^2 and placed between two copper plates. The samples were immediately plunge-frozen in liquid propane at -180 °C using a KF80 plunge freezing unit (Reichert Jung, Vienna, Austria). The frozen samples were kept in liquid nitrogen until further processing. Before fracturing, the samples were placed in a sample holder allowing a longitudinal fracturing parallel to the adhesive strip. The sample holder was transferred into a Balzers BAF 400-D freeze fracture device. Fracturing took place at -150 °C under a pressure of 2.10^{-4} Pa, which was immediately followed by evaporation of platinum at an angle of 45° and evaporation of carbon at an angle of 90° resulting in subsequent layers of 3 nm platinum and 4 nm carbon on the freshly obtained fracture plane. The thus produced replicas were removed from the freeze fracture device and cleaned by submersion in 0.5 M

quaternary ammonium hydroxide in toluene (Soluene TM-350, Packard, Groningen, The Netherlands) for three days. After this period, the replicas were rinsed three times with toluene. The replicas were mounted onto 400 mesh copper grids (Balzers, Liechtenstein) and visualised using a Philips EM410 (Philips, Eindhoven, The Netherlands) transmission electron microscope. On average, 60 electron micrographs (15 of each tape-strip) were taken for each moisturiser or the control site.

These micrographs were assessed and visually scored for several characteristics by three independent investigators. At least 40 micrographs were analysed per moisturiser.

In order to have a means of identifying the moisturisers on the micrographs, replicas of neat moisturiser as well as moisturiser saturated with water were prepared. Moisturiser saturated with water was prepared by mixing it vigorously with equal amounts of water shortly before plunge freezing. A drop of neat moisturiser or moisturiser saturated with water was placed between copper preparation holders. Freezing, fracturing and platinum and carbon evaporation were performed in the same way as for the tape-strip samples.

Isolation of ceramides

Fresh pig skin was obtained from a slaughter house. The skin surface was briefly washed with 70% ethanol to remove the surface lipids. Hair was removed with a scalpel. The skin was dermatomed to a thickness of approx. 300 µm and soaked overnight in a 0.1% trypsin in phosphate buffered saline (pH 7.4) at 4°C. After incubation at 37°C for 1h, the SC could be removed from the viable epidermis mechanically. After placing the SC sheet in 0.1% trypsin inhibitor in water for 30 minutes, the SC was washed twice with water, placed on a small-mesh wire net, and dried to the air. The lipids were then extracted from the obtained pig SC according to Bligh and Dyer (Bligh and Dyer, 1959),dissolved in chloroform-methanol 2:1 and stored at -20°C. Subsequently, the extracted lipids were applied on a Silicagel 60 (Merck) column with a diameter of 2 cm and a length of 20 cm and eluted using various solvent mixtures as described previously (Bouwstra et al., 1996a). The eluted lipids were collected in 3-ml fractions. The lipid composition of individual fractions was established by one-dimensional high performance thin layer chromatography, as described before (Ponec et al., 1988). For quantification, authentic standards (Sigma) were run in parallel. The quantification was performed after charring using a photodensitometer with automatic peak integration (Desaga, Heidelberg, Germany). Fractions that only contained ceramides were combined in order to obtain the ceramide mixture for preparing the lipid mixtures. This ceramide mixture

contained approx. 12 % of the acylceramide CER1, whereas the remainder is made up of the porcine short chain ceramides 2 – 6, 2 being the most abundant.

Preparation of skin lipid mixtures

For the preparation of the four-component-mixtures (cholesterol (CHOL):ceramides (CER):free fatty acids (FFA):moisturiser, in molar ratio 2:1:1:1), the following FFA mixture was used: C16:0, C18:0, C20:0, C22:0, C23:0, C24:0 and C26:0 at molar ratios of respectively 1.3, 3.3, 6.7, 41.7, 5.4, 36.8 and 4.7(Wertz, 1991). Appropriate amounts of individual lipids dissolved in chloroform:methanol 2:1 (v/v) were combined to yield lipid mixtures of approximately 1.5 mg total weight at the desired composition with a concentration of 7 mg/ml. A Camag Linomat IV was used to apply the lipid mixtures on mica. This was performed at a rate of 4.3 µl/min under a continuous nitrogen flow. After drying, the samples were equilibrated for 10 min at 60 °C and subsequently hydrated with an acetate buffer of pH 5.0. The lipid weight of the samples was approximately 1.5 mg. Finally, the samples were homogenised by 10 successive freeze–thawing cycles between –20 °C and room temperature, during which the samples were stored under gaseous nitrogen.

Preparation and pre-treatment of in vitro SC samples

Human SC was obtained from cosmetic surgery. The skin was dermatomed to a thickness of 250 µm approx. and the SC was isolated and stored as described above.

A SC strip of 70 by 5 mm was rolled up and pressed to flatten, resulting in a tight 'stack' of approx 45 SC layers. The stacks were stored under argon on silica gel. Prior to the X-ray diffraction measurement, the dry SC stacks were completely immersed in neat moisturisers for a period of 24 h at 32°C.

Small angle X-ray diffraction

All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) using the Dutch-Belgian beamline (BM26B). A more detailed description of this beamline has been given elsewhere (Bras, 1998). The X-ray wavelength and the sample-to-detector distance were 1.24 Å and 1.7 m, respectively. Diffraction data were collected on a two-dimensional multiwire gas-filled area detector. The spatial calibration of this detector was performed using silver behenate and cholesterol. The samples were mounted in a specially designed sample holder with mica windows. All measurements were recorded at room temperature for a period of 10 min.

SAXD provides information about the larger structural units in the sample, such as the periodicity of a lamellar phase, which is the distance over which the structure is repeated. The dimensionless scattering intensity (I) is measured as a function of the scattering vector q (in nm^{-1}). The latter is defined as $q = 4\pi \sin \vartheta/\lambda$, in which ϑ is the scattering angle and λ is the wavelength of the X-rays. From the positions of a series of equidistant peaks (q_n), the periodicity d of a lamellar phase is calculated using the equation $q_n = 2n\pi/d$, n being the order number of the diffraction peak.

4. Results

Freeze fracture electron microscopy

Tape-stripping depth as determined by TEWL: After application, the moisturisers behave differently. Both ISIS and IPIS migrated outward from the application site, parallel to the skin surface, leaving only part of the moisturiser on the application site. The more viscous GMIS however, remained on the application site as a thick layer. TEWL values measured before each tape-strip are shown in figure 1. Tape-strip 1 corresponds to the skin surface, while tape-strips 5, 15 and 25 depict the deeper layers of the SC. TEWL increased from 5-6 $g.m^{-2}.h^{-1}$ before tape-stripping to 9-11, 18-20 and 46-59 $g/m^2.h^{-1}$ respectively for tape-strip 5, 15 and 25, with very little variation between control and treated sites.

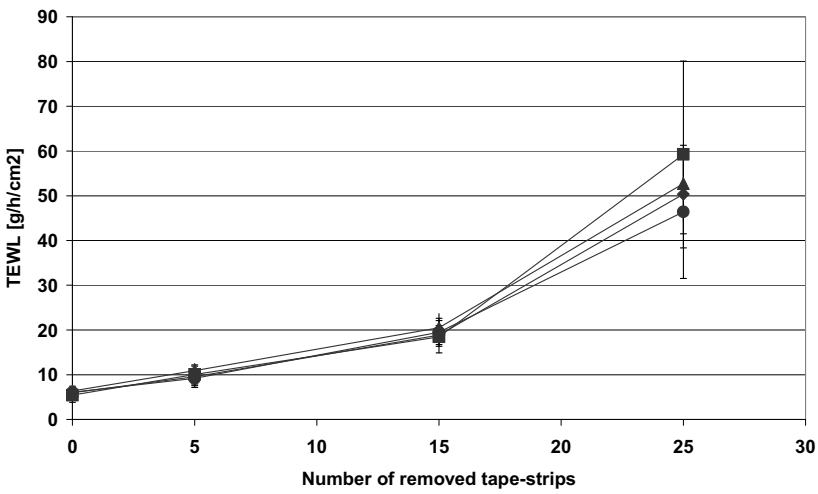

Figure 1. TEWL Values as a function of the number of tape-strips removed. ♦Control, ■ ISIS treatment,▲ IPIS treatment and ● GMIS treatment. Each value represents the mean of four volunteers SD. No difference in TEWL values was observed between the control and the treatments.

Visualisation of moisturisers: As a control, replicas of dry (neat) moisturisers were prepared. The fracturing of neat moisturiser appeared very difficult and fracture planes were only very

rarely obtained. Instead, the fracturing seemed to take place on the interface of the moisturiser and the sample holder (not shown). Water saturated moisturiser was used as a second control. The appearance of ISIS and IPIS to the naked eye did not change after saturation with water. GMIS, however, self-emulsified, resulting in a thick white emulsion. Freeze fracturing of hydrated moisturisers was successful. Micrographs of replicas are shown in figure 2. Images of hydrated ISIS (fig 2a) showed bolt-shaped edges, indicating an ordered structure in the moisturiser. Besides this structure, rough regions were observed, in which the bolt-shaped edges were almost absent. These rough regions indicating an amorphous structure were more apparent in replicas of hydrated IPIS (fig 2b) than in replicas of hydrated ISIS. Replicas of the emulsion formed by GMIS (fig 2c) showed smoother surfaces with many spherical structures of different sizes.

Interaction between moisturisers and the intercellular lipid matrix: In figure 3, micrographs of the replicas of the control site are shown. On all images, intercellular lipids are visible as smooth fracture planes. On images obtained from tape-strip 1 (fig 3a), corneodesmosome remnants, visualised as oval to round areas of small dots, are visible, indicating a fracture across a corneodesmosome. On the images of tape-strips 5 (fig 3b), round, almost intact corneodesmosomes are found next to partially degraded corneodesmosomes, which have been fractured straight through.

In the deeper layers of the SC (tape-strip 15 and 25, figures 3c and 3d) predominantly intact corneodesmosomes are visualised, although degraded corneodesmosomes remain visible until at least tape-strip 15. The corneodesmosomes are surrounded by smooth lipid planes. The lamellar structure of the lipid regions becomes visible when they are fractured perpendicular resulting in sharp edges, referred to as 'lipid steps', see figure 3c and 3d.

On fracture planes resulting from the tape-strips collected from the moisturiser application sites, there is no difference in the appearance of the SC lipid regions, compared to the control sites (not shown). Corneodesmosomes and corneodesmosome remnants, as well as fractures across lipid lamellae are frequently observed as round structures and sharp edges, respectively. However, additionally, fractures across moisturiser regions located in separate domains become apparent.

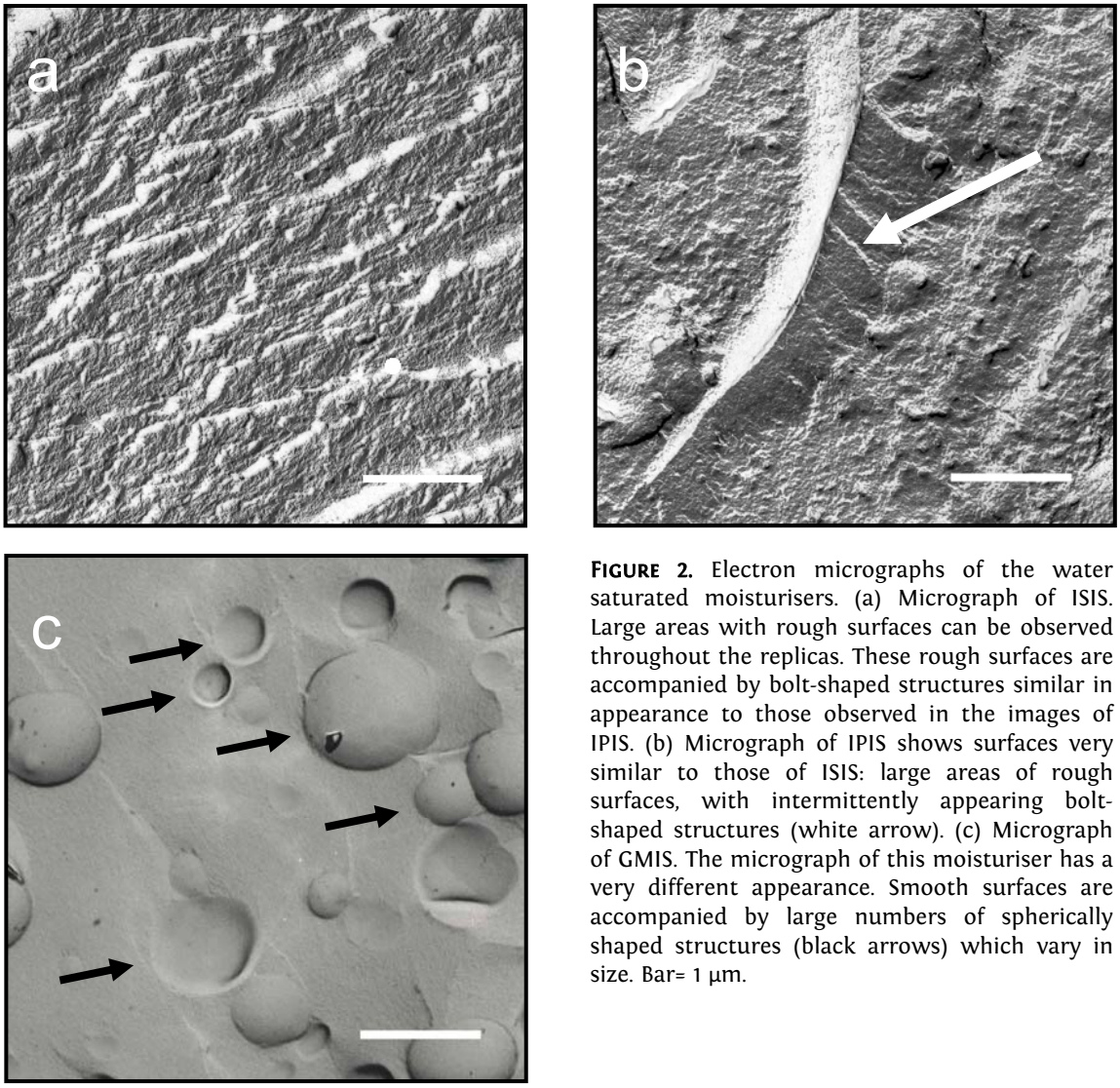

Figure 2. Electron micrographs of the water saturated moisturisers. (a) Micrograph of ISIS. Large areas with rough surfaces can be observed throughout the replicas. These rough surfaces are accompanied by bolt-shaped structures similar in appearance to those observed in the images of IPIS. (b) Micrograph of IPIS shows surfaces very similar to those of ISIS: large areas of rough surfaces, with intermittently appearing bolt-shaped structures (white arrow). (c) Micrograph of GMIS. The micrograph of this moisturiser has a very different appearance. Smooth surfaces are accompanied by large numbers of spherically shaped structures (black arrows) which vary in size. Bar= 1 μm.

The appearance of the regions of phase-separated moisturizer on tape-strips is somewhat different from that of water-saturated moisturisers. The domains of ISIS (fig 4a, 4b) show bolt-like edges, which have some similarities with the forks characteristic for fracture planes across water (vanHal et al., 1996), but appear less sharp in the fracture plane of moisturiser domains. These fine edges are also seen on fracture planes of IPIS (fig 4c, 4d), although in some images they seem to be much longer in this moisturiser and are located in a similar direction indicating a certain degree of orientation of the moisturisers. The edges are different from the sharp steps, which are often observed in lamellar phases (figures 3b and 3d). The appearance of GMIS domains (fig 4e, 4f) in SC is different from that of the other moisturisers. The fracture planes across the moisturiser domains reveal rough surfaces, showing a granite-like structure. Sharp edges are not observed; instead, the surface is parted by large grooves. Frequently, small, spherical structures similar to but smaller than those observed in hydrated

GMIS, can be observed in the moisturiser domains. Interestingly, in several cases these spherical particles were located in the so called channel-like-structures, also observed in previous studies. (see figure 4f) (Honeywell-Nguyen et al., 2003a).

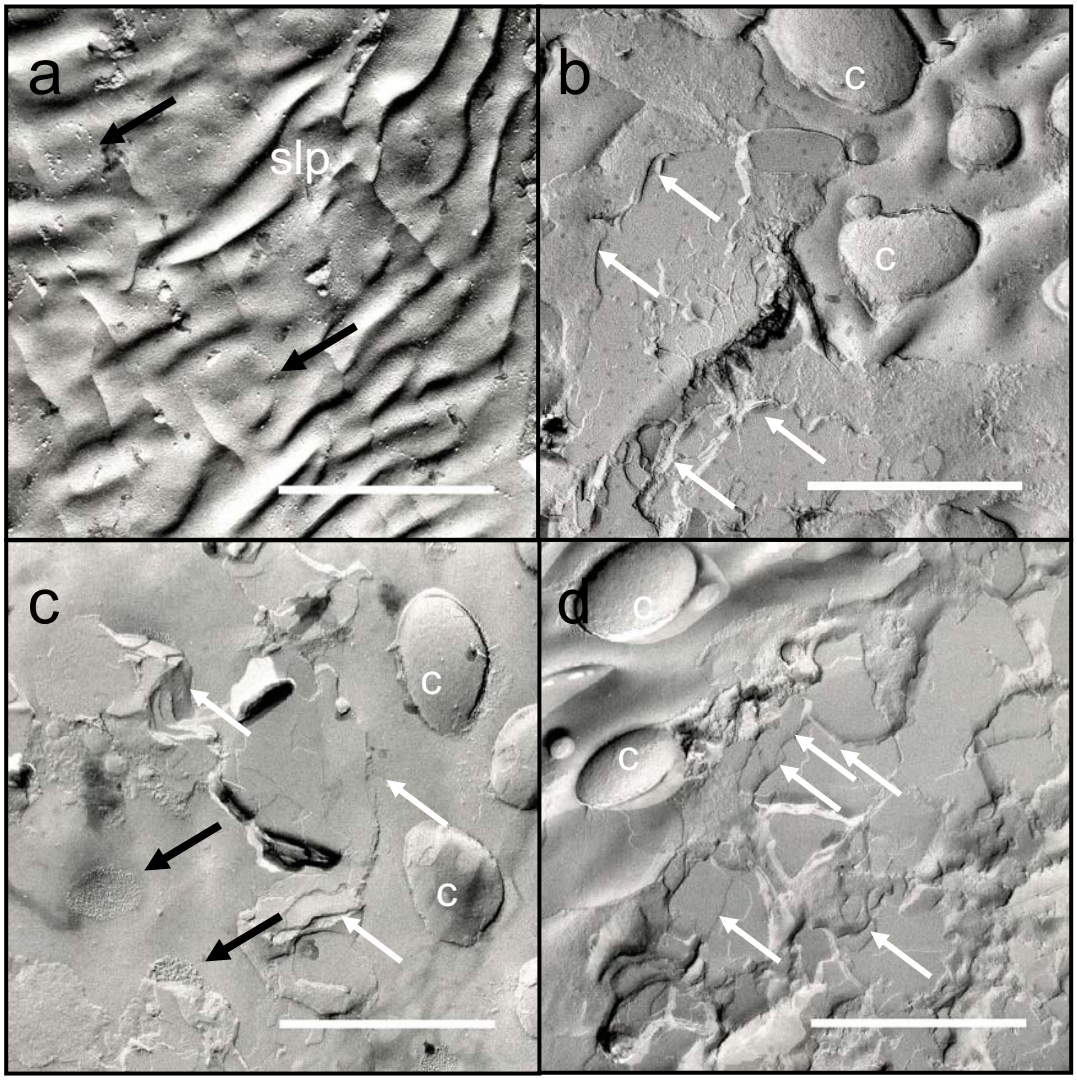

Figure 3. Electron micrographs of replicas from the control site. (a) Micrograph of tape-strip 1. On the first tape-strip smooth surfaces representing lipid domains are observed. Within these lipid domains many degraded corneodesmosomes (corneodesmosome remnants) are visible. (b) Micrograph of tape-strip 5. Moving further into the SC, lipid steps become apparent. These indicate a fracture plane across, rather than along the lipid lamellae. Also, less degraded corneodesmosomes are visible. (c) Micrograph of tape-strip 15. At this depth, intact corneodesmosomes become more abundant, although corneodesmosome remnants are still frequently observed. Lipid steps, i.e. fractures through lipid layers, are also observed more often. (d) Micrograph of tape-strip 25. At this depth, the number of corneodesmosome remnants has markedly decreased. Smooth lipid surfaces and lipid steps are still observed. black arrows=corneodesmosome remnants, c=intact corneodesmosomes, slp=smooth lipid surfaces, white arrows=lipid steps. Bar= 1 µm.

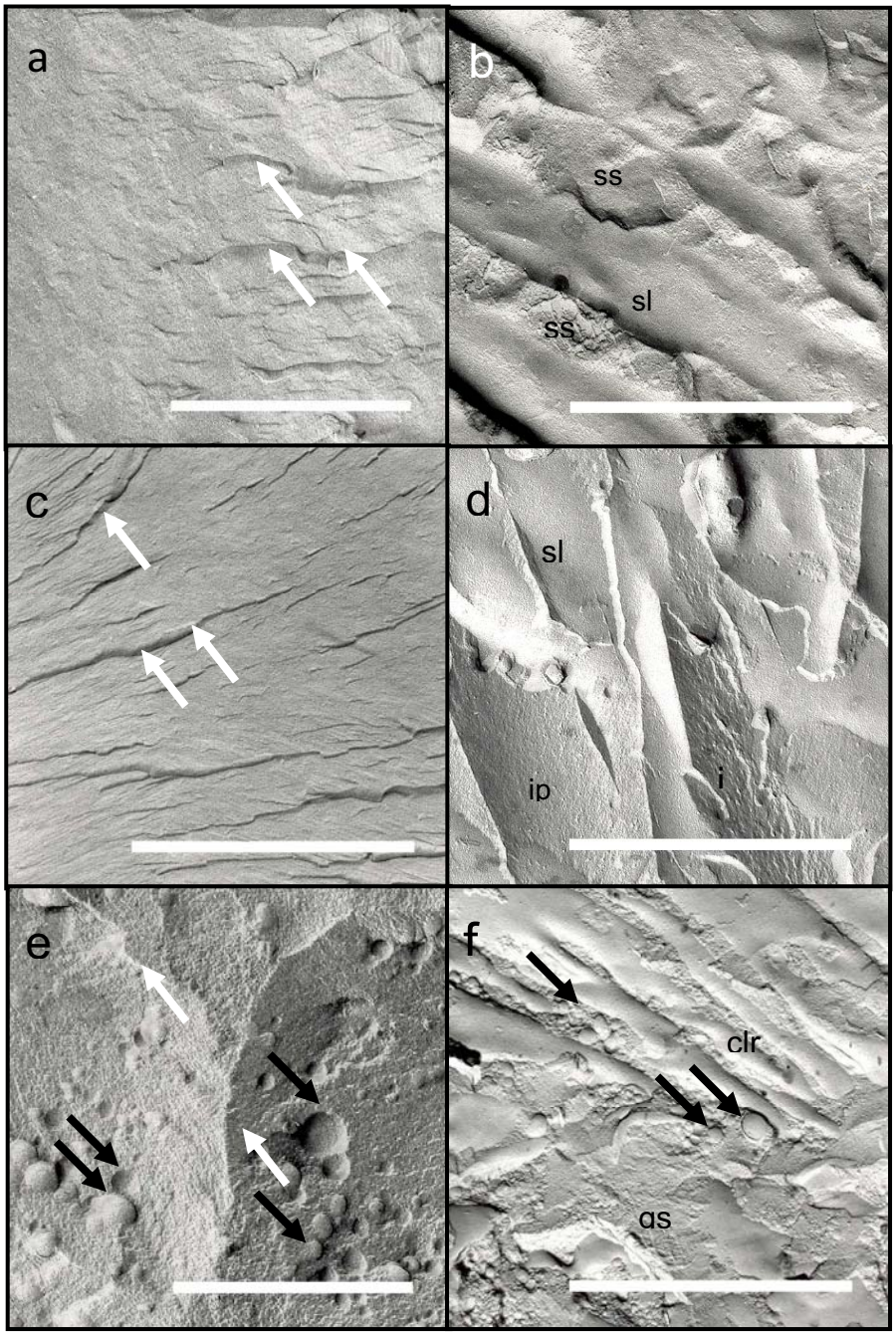

Figure 4. Typical examples of electron micrographs of replicas obtained from the application site. (a,b), Micrograph of ISIS (ss)treated site: tape-strip 15 and 15, respectively. The visualised moisturiser shows edges, similar to those that have previously been described for water (so-called 'forks'), but a less sharp appearance (see white arrows). ISIS is also observed in large plaques on the smooth lipid surfaces of the SC. This is observed in the images of the 15th tape-strip. (c,d) Micrograph of IPIS (ps) treated site: tape-strip 15 and 1, respectively. Edges are observed similar to those in ISIS. However, in images of this moisturiser, the distances between edges are smaller than in case of ISIS (c).. (e,f) Micrograph of GMIS (gs) treated skin: tape-strip 1 and 5, respectively. GMIS treated site has a different appearance. In large plaques many spherical structures are visible. In micrographs of the 5th tape strip the spherical structures appear in so-called 'channel-like-regions' (see micrograph f) and are also observed in the 15th tape strip, though less frequently. White arrows=edges, black arrows=spherical structures, clr=channel-like-regions.

In order to provide a semi-quantitative indication of the frequency of appearance of the several entities described above, the micrographs were scored. The scoring included the presence of smooth lipid surfaces, steps indicating fractures across lipid lamellae, corneodesmosomes, corneodesmosome remnants, and rough structures indicating the presence moisturiser. Additionally, micrographs were scored for the presence of spherical structures in the moisturiser or in channel-like-structures. The scoring is presented in table 1. The results of this scoring, which show some interesting trends, are summarised below.

1 Extracellular lipid, either smooth or cross-fractured (lipid steps) are observed on replicas of all tape-strips except tape-strip 1 of the GMIS application site, indicating the abundant presence of this moisturiser on the first tape strip.
2 Images obtained from the ISIS or IPIS application sites revealed a small reduction in the frequency in which SC lipid regions are visualised compared to the images from the control site, especially in images obtained from the first and fifth tape strip. However, for GMIS the lipid surfaces were much less frequently observed in the images obtained from the first and fifth tape-strip.
3 A similar trend is observed for corneodesmosomes and their remnants for the control and ISIS and IPIS treated sites. On images of the first tape-strip intact corneodesmosomes are rarely seen. However, from the 5th tape-strip on, both corneodesmosomes and remnants are observed. In the case of GMIS intact corneodesmosomes are only infrequently observed until the 25th tape strip.
4 The moisturisers are frequently observed on micrographs of treated sites. Rough structures are most frequently observed in images of tape-strips of the GMIS treated site. In the images obtained from the treated site of one of the volunteers, regions of phase-separated IPIS are more frequently observed in images of tape-strip 15 than of tape strips 1 and 5.

Small angle X-ray diffraction analysis of sprayed skin lipid/moisturiser mixtures

The peak height distributions of the 2nd and 3rd order peak height distributions, as well as average spacings and corresponding periodicities d (n=3) are summarized in table 2. Representative diffraction curves are shown in figure 5. From the diffraction profiles of the control samples (CER:CHOL:FFA, 2:1:1) three phases can be distinguished. Firstly, 1st - 8th order diffraction peaks can all be attributed to a lamellar phase with a periodicity of approximately 13 nm, referred to as the long periodicity phase (LPP)(Bouwstra et al., 2002a). Of this diffraction profile the 1st, 2nd and 3rd order peaks are the most prominent ones. In addition, one low-

intensity peak is observed at q = 1.24 nm^{-1}, attributed to a 2nd phase. This may indicate the formation of a lamellar phase with a periodicity of approximately 5.4 nm, which has frequently been observed in previous studies (Bouwstra et al., 1998). Additionally, a reflection at q = 1.87 nm^{-1} is observed, indicative for phase separated cholesterol. As a further control, samples of liquid, neat moisturisers were measured under equal settings and conditions (data not shown). In the diffraction profiles of ISIS and IPIS no peaks were observed. In the diffraction profile of GMIS, however, one low intensity diffraction peak at q = 1.87 nm^{-1} is visible, the same value as phase separated cholesterol. In the diffraction profiles of lipid mixtures prepared with moisturiser and skin lipids several changes become apparent.

1. Most importantly, the reflection at 1.24 nm^{-1} indicating the presence of the SPP is not detected. This may signify that the three moisturisers prevent the formation of the SPP.
2. The reflections attributed to the LPP shift slightly to lower q-values after addition of IPIS or GMIS, indicating an increase in the periodicity of the LPP. For IPIS the periodicity increased slightly from 13.2 to 13.4 nm, whereas the more hydrophilic GMIS resulted in an increase to 13.8 nm. After addition of ISIS, no change is observed.
3. The intensity distribution of the 2nd and 3rd order peaks of the LPP changes, after addition of GMIS. The intensity of the 3rd order peak increases compared to the 2nd order diffraction peak.
4. In the diffraction peak of GMIS a broad peak at 1.87 nm^{-1} was observed. This peak was not observed in the diffraction pattern of the 2:1:1:1 cholesterol:ceramide:FFA:GMIS lipid mixtures indicating that no large amounts of GMIS phase separated in the lipid mixture.

The increase in the periodicity of the LPP and the change in intensity distribution of peaks attributed to the LPP both indicate that IPIS and GMIS are to some extent integrated into the LPP structure.

Small angle X-ray diffraction analysis of immersed SC samples

Representative diffraction curves of the samples of SC stacks aligned parallel to the primary beam are shown in figure 6. Untreated SC shows the 1st, 2nd, 3rd and 4th order diffraction peaks of the LPP, of which the 2nd order peak is the most prominent. Most probably, the SPP's 1st order peak may well be concealed by the 2nd order diffraction peak of the LPP, which has a similar q-value as can be inferred from the large width of this peak.

In the diffraction patterns of treated SC, only very few changes are observed compared to the control (i.e. untreated SC). Only in ISIS treated SC some changes are observed. In two out of three SC donors the intensity of the 1st order peak of the LPP of the samples treated with ISIS was stronger than the same peak in the patterns of the control samples. This suggests that ISIS might to some extent be incorporated in the lipid lamellae of the LPP of isolated SC, but these changes are only minor. No changes in the periodicity of the LPP were observed.

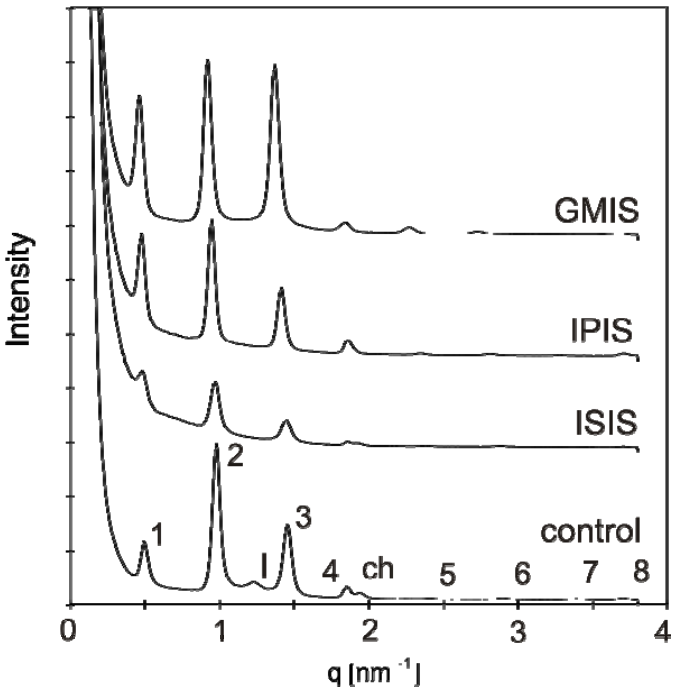

Figure 5. Effect of the addition of 20% moisturiser to the ceramides:free fatty acids:cholesterol mixtures (2:1:1 molar ratio). Orders of the diffraction peaks attributed to the LPP are indicated by Arabic numbers, those of the SPP by Roman numbers. In the diffraction curve of the control sample, 8 diffraction peaks of the LPP are visible, and one of the SPP. Also, a small peak of phase separated cholesterol can be seen at 1.87 nm^{-1} can be seen. Addition of 20% moisturiser results in the disappearance of the 1st order diffraction peak of the SPP. Furthermore, changes in the intensity distribution of the diffraction peaks attributed to the LPP are noticed, which is most prevalent after addition of GMIS.

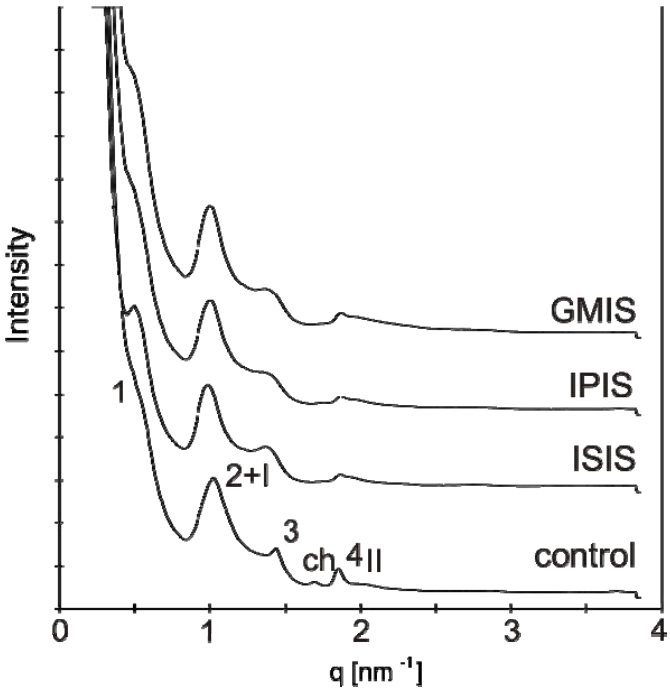

Figure 6. Typical examples of diffraction curves obtained of SC pre-treated with moisturiser for 24 hours at 32 C. Diffraction peaks attributed to the LPP are indicated by Arabic numbers, those attributed to the SPP by Roman numbers. The diffraction curves of the control (untreated stratum corneum) and of the pre-treated SC are very similar, showing that the pre-treatment does not affect the lamellar phases in SC.

Table 1 :Scoring of the freeze fracture electron micrographs:

Treatment	Tape strip	No. of pictures taken	Smooth lipid surfaces present	Lipid steps present	Corneosomes	Moisturiser present	Spherical structures (total)	Spheres in channel-like-structures
control	1	25	+++	++	r	n/a	-	-
	5	13	+++	++	c/r	n/a	-	-
	15	14	+++	++	c/r	n/a	-	-
	25	17	+++	++	c/r	n/a	-	-
ISIS, 3 h	1	30	++	++	r	++	-	-
	5	15	++	++	c/r	+	-	-
	15	27	++	+	c/r	++	-	-
	25	19	++	++	c/r	++	-	-
IPIS, 3 h	1	46	+++	++	r	++	-	-
	5	32	++	++	c/r	+	-	-
	15	19	+	+	-	+++	-	-
	25	17	++	++	c/r	+	-	-
GMIS, 3 h	1	46	-	-	r	+++	++	++
	5	57	+	+	r	+++	+	+
	15	31	++	+	r	+++	+	+
	25	11	+++	++	c/r	+	-	-

- seen in less than 10% of the pictures
+ seen in 10 to 40% of the pictures
++ seen in 40 to 70% of the pictures
+++ seen in more than 70% of the pictures
r there are at least 3 times as many pictures with corneodesmosome remnants than with corneosomes
c there are at least 3 times as many pictures with corneosomes than with corneodesmosome remnants
c/r there are less than three times as many pictures with corneosomes or corneodesmosome remnants

Table 2: Peak height distribution, spacings q and corresponding periodicities d.

Values are averages (n=3) standard deviation.

	Peak height distribution	Peak position q of the 2nd order diffraction peak of the LPP [nm^{-1}] *	Repeat distance d [nm] *
Control	2 > 3	0.95 0.03	13.3 0.4
20% ISIS	2 > 3	0.95 0.02	13.2 0.2
20% IPIS	2 > 3	0.94 0.01	13.4 0.1
20% GMIS	3 > 2	0.91 0.01	13.8 0.1

5. Discussion

Cosmetically dry skin and dry skin caused by dermatological disorders is often treated by the application of lipophilic moisturisers. It is sometimes speculated that these moisturisers penetrate the skin or even enter the lipid domains in the SC. The latter speculation causes some concern, as changes in lipid composition might have deleterious effects on the lipid organisation in the SC, which is essential for correct functioning of the skin barrier. In this study, the effect of three lipophilic moisturisers on the SC structure was studied using two methods: FFEM, which offers the possibility to visualise the SC morphology in vivo, and SAXD, which provides information on the effect of moisturisers on the lipid lamellar phases in more detail in vitro.

Freeze fracture electron microscopy

Tape-stripping depth as determined by TEWL: TEWL values are often used in tape-stripping studies to give an indication of the depth to which the SC has been removed. During our studies we observed a difference in lateral diffusion of ISIS and IPIS on the one hand and the lack thereof for GMIS on the other. As excess moisturiser was not removed prior to tape-stripping, this difference could mean the tape-stripping depth of the GMIS application site would lag behind approximately 1 to 5 tape-strips. However, the TEWL values measured at this application site did not confirm this, suggesting that either the TEWL measurements were not sensitive enough to monitor this difference in stripping depth or that GMIS influences the TEWL values themselves, by increasing the water evaporation from the SC. Considering the observations made after application, GMIS may occlude the skin to a certain extent, thereby retaining water in the SC, but this could not be confirmed by TEWL measurements for the reasons described above.

Visualisation of the moisturisers: At physiological temperatures, the three neat (dry) moisturisers used have very similar appearances. Unfortunately, this could not be confirmed microscopically, because freeze fracturing neat moisturiser proved to be impossible.

When saturating the moisturisers with water, the difference between their molecular structures became apparent: GMIS emulsified into a thick, white fluid, which is stable for several days, whereas ISIS and IPIS phase separated. As expected, this difference between the moisturisers was also visible after freeze fracturing. While ISIS and IPIS showed many bolt-shaped structures, GMIS sometimes showed more even, but granite-like fracture planes.

However, GMIS was mostly visualised as smooth surfaces with many spherical structures, as can be seen in figure 2c.

Interaction between moisturisers and the intercellular lipid matrix in vivo: The scoring of micrographs for the observation of smooth lipid surfaces, lipid steps, corneodesmosomes, moisturiser, spherical structures and channel-like structures revealed several observations:

1 After moisturiser treatment, smooth lipid surfaces and lipid steps are visualised less frequently, especially in tape-strip 1 and 5, indicating that moisturiser and lipid domains coexist in the superficial regions in the SC. With respect to the frequency of appearance of the smooth lipid surfaces and lipid steps, no difference was observed between sites treated with ISIS or IPIS. However, micrographs from GMIS-treated sites revealed almost no lipid surfaces or lipid steps in tape strip 1 and 5. This demonstrates that a large amount of GMIS is present on the skin surface or superficial SC regions and that even at tape-strip 5, mainly GMIS is observed. This has been confirmed by the appearance of the corneodesmosomes, as discussed below.

2 No difference in the in-depth distribution of corneodesmosomes and their remnants between the control and the application sites of ISIS and IPIS were observed. However, after application of GMIS, intact corneodesmosomes are not found until tape-strip 25. This may be caused by either the surplus of GMIS on the skin, resulting in a reduced tape-stripping depth or by a degradation of the corneodesmosomes facilitated by the moisturiser. A definite answer can only be given after the actual penetration depth of the moisturiser has been determined. For this purpose, further experiments have to be performed.

3 In a previous study the channel-like regions mentioned above have been suggested to be folds in the corneocyte envelope surrounding the corneocytes and it was shown that these channel-like regions are the preferred route for intact elastic vesicle permeation. It has been suggested that the lamellar lipid stacks are less organised in the narrow folds, and are therefore providing a reduced barrier for permeation than the surrounding lipid domains (Honeywell-Nguyen et al., 2003a). Interestingly, in this study spherical structures, which may be vesicular structures formed due to the presence of the glycerol group, are also observed in channel-like regions, after application of GMIS, even in deeper regions (tape strip 15) of the SC. If these spherical structures contain water, they could potentially carry water into the deeper layers of

the SC and retain it there. It must be noted however, that the actual tape-stripping depth is uncertain.

Small angle X-ray diffraction analysis of sprayed skin lipid/moisturiser mixtures and pre-treated SC in vitro

The diffraction curves of the control lipid mixtures containing ceramides, free fatty acids and cholesterol (2:1:1) are similar to those in previous studies (Bouwstra et al., 1996a), (Bouwstra et al., 1998). This concerns both the formation of the LPP and the SPP, as well as the peak intensity distribution.

Addition of 20% moisturiser to the lipid mixture reduces the formation of the SPP, while the formation of the LPP is promoted. This is a very interesting observation, as it is proposed that the LPP is characteristic for SC, and contributes to its barrier function in a greater extent than the SPP. In a previous study it has been demonstrated that a certain degree of fluidity is required for the formation of the LPP (Bouwstra et al., 2002b). If the degree of fluidity is too high or too low, the formation of the LPP is reduced in relative terms. The used moisturisers are fluid at room temperature as well as at physiological temperatures and may therefore influence the LPP formation. Whether fluidity plays a role in the formation of the LPP in the presence of the moisturisers is not know yet and will be a subject for further studies.

From the diffraction curves obtained in this study it is clear that the presence of IPIS or GMIS increases the periodicity of the LPP and that GMIS changes the intensity distribution of the various diffraction peaks attributed to the LPP. This clearly establishes that these moisturisers are at least partially incorporated into the LPP. However it does not provide information on the position of the moisturisers *in* the lipid layers. This may also be different for ISIS and GMIS, as GMIS has a hydrophilic group that may function as a head group, whereas ISIS has none. No changes are observed after addition of ISIS. However, this does not mean that this moisturiser is not incorporated into the structure, as it may be incorporated without having an effect on the periodicity of the lipid lamellae formed. Other methods, such as FTIR, may clarify this possibility. It has to be remarked that not only incorporation of moisturiser into the SC lipids is a possibility in these experiment. Using SAXD, it was not possible to detect phase-separated moisturiser. If, however, some of the moisturiser did exist in phase-separated regions, it is possible that SC lipids are extracted and solubilised in these regions, hereby changing LPP and SPP. However, extraction of ceramides or FFA would result in a smaller periodicity, rather that a larger one which was observed in this study. Extraction of FFA would

only have a small effect, whereas no effect is expected from cholesterol extraction (Bouwstra et al., 1996a; Bouwstra et al., 1996b).

The experiments discussed above provided ideal circumstances for SC lipids and moisturiser to form lipid phases together. However, *in vivo* moisturiser must penetrate already existing lipid lamellae. To closely simulate this situation, isolated SC was pre-treated with neat moisturiser for 24 hours. The diffraction curves of dry SC (control) are very similar to those presented in previous studies (Bouwstra et al., 1991) and to the sprayed lipid mixtures. The first four orders of the LPP are clearly visible, but the diffraction peaks are rather broad, which may render the diffraction peaks of the SPP more difficult to detect. This low resolution is caused by the limited number of repeating lipid layers between the corneocytes in isolated SC, compared to that in the sprayed lipid samples. It has been reported that the maximum number of repeating units in the extracellular regions is only twelve, while three and six are the most common (Swartzendruber et al., 1995a).

After immersion of the dry SC samples in moisturiser for 24 hours, only very few changes are visible. The four peaks attributed to the LPP are still visible, whereas the SPP remains hidden. There are no substantial changes in peak height distribution, although the 1st order peak of the LPP seems to become somewhat stronger after immersion in ISIS. This obviously shows that the moisturisers only minimally change the lipid organisation in SC.

Conclusions

Summarising, the results clearly show that the three examined lipophilic moisturisers have no disordering effect on the SC lamellar organisation, as was shown by SAXD measurements, even though is clear that minimally two of the studied moisturiser molecules are able to at least partially penetrate the lipid structure. In contrast, many penetration enhancers such as oleic acid or azones, which also have this ability, have been shown to destroy the lipid lamellae completely (Suhonen et al., 1999a). Whether the penetrating moisturiser molecules change the lateral packing of the lipid lamellae, still remains to be investigated.

The depth to which the investigated lipophilic moisturisers penetrate the SC, largely influences their effect on it. In this study TEWL was used to determine changes in the skin barrier as a function of tape-stripping. However, this did not provide information on the penetration depth of the moisturisers, for which other analysis methods such as ATR-FTIR and SC quantification must be used. These will be a subject of further studies. Nevertheless, the results presented in this study provide information on the effects of the three studied

moisturisers on dry skin. If we extrapolate these findings to dry skin *in vivo* we can make the following speculations. Firstly, that the moisturisers penetrate the top layers of the SC. They are therefore not (exclusively) occlusive in the traditional sense, but may still result in an increased SC humidity below the penetration region. Secondly, this increased humidity may have an effect on the SC processes such as cornification, NMF and desquamation. An activity increase of the enzymes in charge of these processes may therefore result in a decrease of the symptoms of dry skin, and dry skin itself. Thirdly the effect of GMIS may be enhanced by the spherical structures that are visible after *in* vivo application of this moisturiser. If these spheres do indeed contain water, this water may be available to the SC enzymes at some point, and cause the same enzymatic changes explained above. However, further experiments, for instance using FTIR spectroscopy, should be performed in order to further explain the mode of action of lipophilic moisturisers.

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